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Initiation of (−)-Strand RNA Synthesis Catalyzed by the BMV RNA-Dependent RNA Polymerase: Synthesis of Oligonucleotides

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RNA replication, a process of fundamental importance for pathogenesis by many viruses, remains poorly understood at the mechanistic level because relatively few of the responsible enzymes have been purified and characterized biochemically. Partially purified RNA-dependent RNA polymerase (RdRp) from brome mosaic virus (BMV)-infected barley leaves is able to synthesize (−)-strand RNAs from input (+)-strand templates. In resolving RdRp products generated during (−)-strand BMV RNA synthesis, we found that an RNA of eight nucleotides was generated at approximately 10-fold molar excess to the full-length (−)-strand RNA. Production of the 8-mer was dependent upon and specific to BMV RNA templates. Furthermore, inhibitors of full-length (−)-strand RNA synthesis by RdRp also affected the production of the 8-mer. Analysis of the sequence of the 8-mer suggests that it is initiated at the authentic initiation site on the BMV RNA template, consistent with the possibility that the 8-mer is an abortive initiation product of RNA synthesis by RdRp *in vitro*. Addition of heparin or Mn^{2+} differentially affected production of the 8-mer and full-length (−)-strand RNA, as did some nucleotide changes near the site of RNA initiation. These studies further define the initiation process of (−)-strand RNA synthesis.

INTRODUCTION

RNA-dependent RNA polymerase (RdRp) is the enzyme responsible for the synthesis of RNA from an RNA template. While many sequences for viral RdRp proteins have been determined (Ishihama and Barbier, 1994), RNA synthesis by RdRp is relatively poorly understood. Recent elucidation of the structure of several polymerases revealed common themes in the mechanism of polymerase activity (reviewed in Joyce and Steitz, 1995). Therefore, further elucidation of RdRp activity can likely use the knowledge accumulated from DNA-dependent RNA polymerases. Synthesis of RNA from a DNA template includes at least six discrete steps: (1) specific binding of enzyme to the promoter; (2) melting of DNA to form an open complex; (3) initiation of RNA synthesis; (4) transition from the initially transcribing complex to an elongating complex; (5) elongation; and (6) termination of RNA synthesis (Carpousis and Gralla, 1985; Krummel and Chamberlin, 1992; McClure, 1985). A key part of this process is that immediately after the initiation of transcription, the polymerase enters a phase in which dissociation of the enzyme–DNA–RNA ternary complex significantly competes with elongation, resulting in the production of oligoribonucleotides. This process was referred to as abortive cycling (Carpousis and Gralla, 1980). "Productive" transcripts are formed only when abortive initiation is overcome by a change in the RNA polymerase. Abortive cycling seems to be an innate property of most DNA-dependent RNA polymerases, including those from T7 bacteriophage (Martin *et al.,* 1988), *Escherichia coli* (Carpousis and Gralla, 1980), and mammalian cells (Ackerman *et al.,* 1983).

We study the mechanism of RNA replication using brome mosaic virus (BMV), a minor pathogen of cereal crops, as a model system (Ahlquist, 1992). The genome of BMV is divided into three separately encapsidated (+)-strand RNAs: RNA1 (3.2 kb), RNA2 (2.8 kb), and RNA3 (2.1 kb) and a subgenomic RNA4 (0.88 kb). At present, the BMV RdRp is known to be composed of at least three polypeptides, the RNA1-encoded 109-kDa 1a protein, the RNA2-encoded 94-kDa 2a protein, and a host protein associated with the translation initiation factor eIF3 (Kao *et al.,* 1992; Quadt and Jaspars, 1990; Quadt *et al.,* 1993). As is common to the (+)-strand RNA viruses, the BMV RdRp complex is associated with cellular membranes, but can be solubilized with high-salt and non-ionic detergents such as Triton X-100 (Miller and Hall, 1983; Kao and Sun, 1996). The BMV RdRp has been highly enriched (Miller *et al.,* 1986; Miller and Hall, 1983; Quadt and Jaspars, 1990; Kao and Sun, 1996) and can specifically synthesize (−)-strand RNA from input (+)-strand RNA template in a sequence-specific manner. In addition, the BMV RdRp can also synthesize subgenomic (+)-strand RNA 4 by initiating internally within the (−)-strand of RNA 3 (Miller *et al.,* 1986). The synthesis of (−)-strand RNA initiates from the conserved 3′ ends of (+)-strand BMV RNAs which can fold into tRNA-like structures (Ahlquist *et al.,* 1981; Rietveld *et al.,* 1983). Initiation of (−)-strand RNA synthesis starts at the penultimate cytidylate, making the

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first residue a guanylate (Kao and Sun, 1996; Miller *et al.,* 1986).

In this communication, we demonstrate that the BMV RdRp generates oligoribonucleotide products during the initiation of (−)-strand RNA synthesis. A major initiation product of 8 nucleotides is present in approximately 10-fold molar excess to elongated RNA products. The steps for generating this 8-mer and elongated products can be separated using different metal ions and inhibitors.

MATERIALS AND METHODS

RdRp preparation

BMV RdRp was prepared from infected barley essentially using the scheme described by Quadt *et al.* (1993). Briefly, RdRp was enriched from membranes of infected barley by sequential passage through columns of Sephacryl S-400 HR (2.5 × 40 cm) and DEAE Bio-GelA (2.5 × 10 cm). A 0.5 *M* KCl eluant from the DEAE Bio-Gel A column was desalted with a gel filtration PD-10 column (Pharmacia Inc.) and used for RdRp assays.

RdRp activity assay

Standard RdRp activity was assayed in 40-μl reactions containing 50 m*M* sodium glutamate (pH 8.2), 10 m*M* $MgCl_2$, 8 m*M* dithiothreitol, 0.4% Triton X-100, 1 μg of template RNA (usually virion RNA unless stated otherwise), and 4 μCi of α-^{32}P-radiolabeled nucleotide (400 μCi/mmol, Amersham Inc.). Unlabeled forms of nucleotides (purchased from Pharmacia Inc.) were included at 1 m*M* final concentrations. After a 90-min incubation, reactions were heated at 90° for 3 min to inactivate RdRp. After cooling the reactions on ice, they were treated with 4 units of alkaline phosphatase (New England Biolabs) for 30 min at 30° in order to decrease the abundance of unincorporated radiolabeled nucleotides. The reaction products were then extracted with a 1:1 mix of phenol and chloroform and precipitated with 3 vol of ethanol and 10 μg of glycogen according to standard protocols (Maniatis *et al.,* 1982).

Electrophoresis and quantification of RdRp products.

Samples were suspended in 1× sample loading dye (5% glycerol, 0.04% bromophenol blue, 0.04% xylene cyanol, and 10 μg/ml of ethidium bromide) for analysis by native gel electrophoresis in 1% agarose–0.5× TBE submerged gels (45 m*M* Tris, 45 m*M* boric acid, 1 m*M* EDTA, pH 8.3). Ethidium bromide was included in the loading dye to decrease artifactual trapping of double-stranded RNA by the agarose gel and to improve resolution (Sun and Kao, 1995). The amount of label incorporated into newly synthesized RNAs was determined using a Phosphorimager (Molecular Dynamics).

For denaturing gel electrophoresis, samples were suspended in 10 μl of formamide loading solution (90% formamide, 0.1% bromophenol blue, 0.1% xylene cyanol). The samples were heated at 90° for 3 min prior to loading a discontinuous slab gel composed of a stacking gel of 5% acrylamide (19:1 acrylamide:bisacrylamide)–7 *M* urea (8 × 14.5 × 0.075 cm) and a resolving gel of 20% polyacrylamide–7 *M* urea (14 × 14.5 × 0.075 cm) according to published procedures (Maniatis *et al.,* 1982). Gels were run at 400 V 1–2 hr before loading of the samples, and then electrophoresis was continued at 400–500 V for 3.5 hr. Following electrophoresis, the gel was wrapped in plastic and autoradiographed at −80°. After visualizing the radiolabeled products, the oligonucleotides of interest were excised with a razor blade using the autoradiogram as a guide and quantified by scintillation counting (Tricarb 2110-TR; Packard Inc.) in 2-ml aliquots of Bio SafeII solution (Research Products International Corp.). In addition, the region from the interphase between the stacking and resolving gel extending down to and including the area containing xylene cyanol dye (ca. 28 nucleotides, Maniatis *et al.,* 1982) was excised and counted. This count will be referred to as the "elongated (E)" RNA products. The molar ratio of 8-mer to full-length (−) RNA was determined using the equation

$$\text{Molar Ratio} = 289 \times (\text{cpm in 8-mer/cpm in E-RNA}).$$

The conversion factor of 289 is the ratio of cytidine in full-length (−)-RNA (an average of 579 cytidylates in the four BMV RNAs) and the two cytidylates in the 8-mer.

Preparation of transcripts.

Transcripts were generated from both plasmids and polymerase chain reaction (PCR) products containing T7 promoters. PCR was used to synthesize cDNA copies of BMV RNA 3 with altered 3′-most sequences. All PCR reactions used a 5′ primer (5′ GCTATGACCATGATTACG 3′) which hybridized to a sequence upstream of the T7 promoter in the cDNA clone of BMV RNA 3, pB3TP8 (Janda *et al.,* 1987). Different 3′ primers were used with the above 5′ primer to generate the following constructs: Wt, Δ1, +1G, +13, mu-1, mu-3, and mu-4. For the Wt cDNA, the 3′ primer sequence was: 5′ TGGTCTCTTTTAGAGATTTACAG 3′. (For this oligonucleotide and the following ones, the underlined G encodes the cytidylate which is responsible for directing the initiation of (−)-strand RNA synthesis.) For Δ1, the primer sequence was: 5′ GGTCTCTTTTAGAGATTTACAGTGTTT 3′. For the +1G, the primer sequence was: 5′ CTGGTCTCTTTTAGAGATTTACAGTGT 3′. For the +13 template the primer sequence was: 5′ ACGCTGGCACTAGTGGTCTCTTTTAGAGATTT 3′. For mu-1, the primer sequence was: 5′ GGGTCTCTTTTAGAGATTTACAGTGT 3′. For mu-3, the primer sequence was: 5′ TGATCTCTTTTAGAGATTTACAGTGTTTTTCA 3′. For mu-4, the primer sequence was: 5′ TGGCCTCTTTTAGAGATTTACAGTGT 3′.

Thirty cycles of PCR were used for amplification, with each cycle consisting of denaturation at 92° for 1 min, annealing at between 43 and 50° for 0.5 min, and elongation at 72° for 2 min. PCR products were purified as described above (Maniatis *et al.,* 1982) and used as template for *in vitro* transcription by T7 RNA polymerase (Ampliscribe, Epicentre Technologies Inc.). After transcription for 2 hr, the DNA template was destroyed with DNaseI, and the newly synthesized RNA was purified twice by centrifugation through Sephadex G-50 spin columns to remove unincorporated NTPs and other small molecules (Maniatis *et al.,* 1982). The transcripts were quantified spectrophotometrically prior to use in an RdRp assay.

Transcripts with three (+GAU) and 17-nucleotide (+17) extensions at the 3′ termini of BMV RNA 3 were generated *in vitro* by transcription from a restriction enzyme-linearized version of the cDNA for RNA3 in a pET12 vector (Novagen Inc.) using the T7 promoter. Transcripts were purified as described above.

Preparation of T7 8-mer.

An 8-mer corresponding to the first 8 nucleotides of (−)-strand BMV RNA was generated using two oligonucleotides and T7 RNA polymerase as described by Martin *et al.* (1988). The nontemplate oligonucleotide contained a 17-nucleotide sequence of the T7 promoter (5′-TAATACGACTCACTATA-3′). The oligonucleotide serving as the template contains the sequence complementary to the T7 promoter and the underlined sequence corresponding to nucleotides −2 to −9 of BMV RNA3 (5′ AAGAGACCTATAGTGAGTCGTATTA 3′).

RESULTS

BMV RdRp generates (−)-strand RNA and oligoribonucleotides

Partially purified BMV RdRp synthesizes full-length (−)-strand RNA *in vitro* from input virion RNAs (Hardy *et al.,* 1979; Miller and Hall, 1983; Quadt and Jaspars, 1990; Quadt *et al.,* 1993). In order to examine the process of the initiation of RNA synthesis, we looked for possible abortive initiation products on a 20% denaturing polyacrylamide gel. In these gels, the newly synthesized full-length (−)-strand RNAs and other large but less than full-length RNAs migrated as heterogeneous products near the top of the 20% denaturing polyacrylamide gel (Fig. 1A). We also observed the synthesis of an 8-mer and several other less prominent oligonucleotides labeled with [^{32}P]CTP (Fig. 1A). Quantitation of the labeled RNAs by liquid scintillation counting revealed that the rate of appearance of the 8-mer and E-RNAs were similar (Fig. 1B). The 8-mer was also observed when labeling was performed with [^{32}P]UTP. In addition, a fainter product of approximately 9 nucleotides was detected (see Fig. 3C). While the production of the 8-mer was observed in all preparations of RdRp tested, the smaller oligonucleotides seen in Fig. 1A were more difficult to reproducibly detect. In some cases they could not be distinguished from the background of unincorporated radiolabel. Thus, we will focus on the 8-mer in the remainder of this report.

The BMV RdRp requires 3 μM CTP for half-maximal activity in the presence of 1 μg of BMV virion RNA (Kao and Sun, 1996). Our labeling reactions contained only 0.3 μM CTP (as [^{32}P]CTP) and were limited for the radiolabeled nucleotide. In order to determine whether limiting amounts of CTP were responsible for the production of the 8-mer, successively higher concentrations of unlabeled CTP were added to the RdRp reactions. The effects of unlabeled CTP on the radiolabeling of both the 8-mer and the E-RNAs were evident in Fig. 1C. Reduced amounts of the 8-mer and the E-RNAs were still produced, even when the CTP concentration was at 30 μM (Fig. 1C, lane 7). Quantitation of the labeled RNAs by scintillation counting showed that the addition of unlabeled CTP affected the synthesis of the 8-mer and E-RNAs in a similar manner, suggesting that the synthesis of the 8-mer was not due to limiting concentrations of CTP (Fig. 1D).

Should the 8-mer be initiated from the same penultimate cytidylate of the virion RNA in the same manner as the synthesis of full-length (−)-strand RNA, then its sequence should be 5′ GGUCUCUU 3′. Experimental support that the 8-mer has this sequence is presented below. Using this sequence, the molar ratio of the 8-mer and E-RNAs in Fig. 1A was quantified. We determined that a slightly higher amount (16-fold) of the 8-mer was present 5 min after the initiation of the RdRp reaction. The ratio dropped to between 8- and 10-fold for all later time points. It should be pointed out that, for this estimation, the E-RNAs were taken to be full-length (−)-strand BMV RNAs and did not allow for the existence of prematurely terminated BMV RNA products. Therefore, estimates of 8–10 molar excesses of the 8-mer represent maximum values.

Production of the 8-mer is template-dependent and specific.

Omitting BMV virion RNA in the RdRp reaction resulted in much reduced amounts of both the 8-mer and E-RNAs (data not shown), suggesting that the 8-mer is generated from BMV RNA. Template specificity for the production of the 8-mer was confirmed and extended by analyzing the products of RdRp reactions containing total tRNA from yeast, 60S rRNA from wheat germ, transcript from the control template (T7C) provided by an *in vitro* transcription kit (Epicentre Technologies), and poly(A-U) RNA (Fig. 2). Two purified tRNAs from yeast, lys-tRNA and trp-tRNA, were also tested (data not shown). In reactions using poly(A-U), radiolabeled UTP replaced [α-^{32}P]CTP. Some low-level

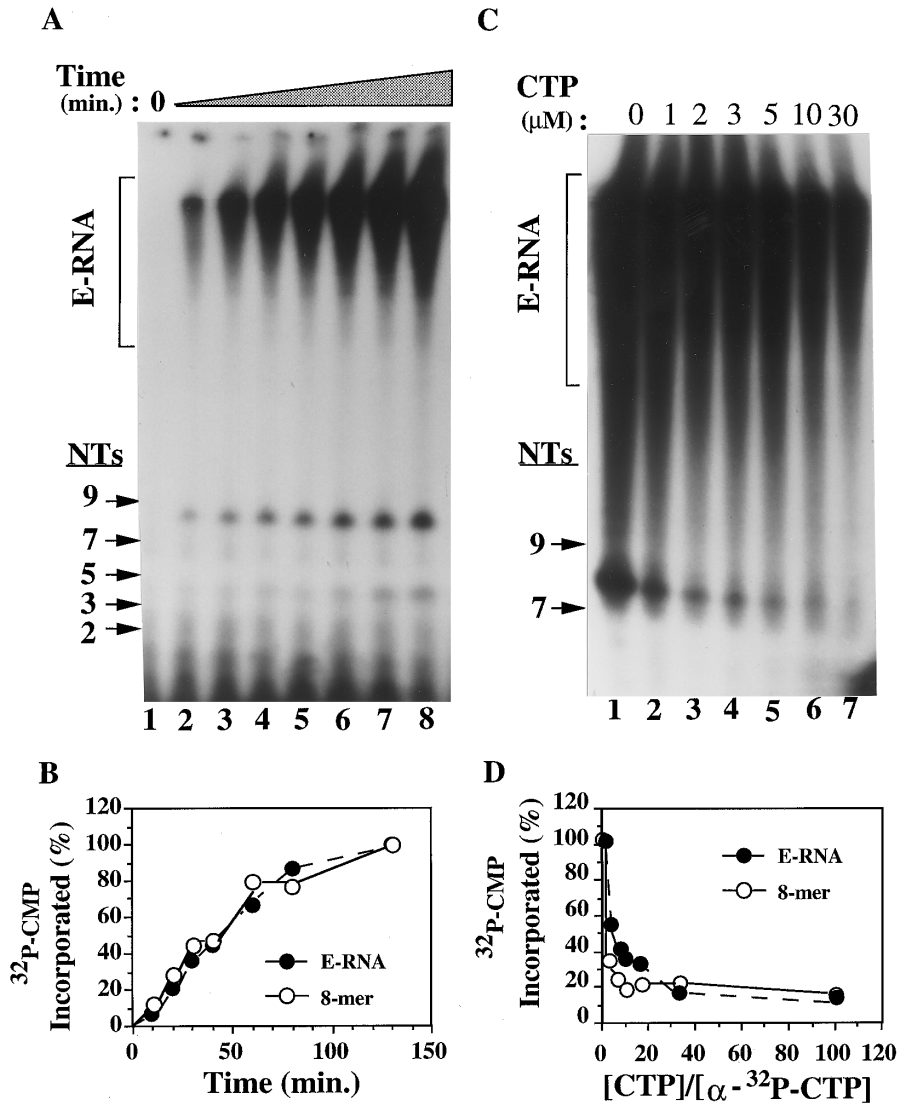

FIG. 1. Synthesis of oligoribonucleotides and longer RNAs. (A) RdRp assays were conducted as described under Materials and Methods and the products were analyzed by electrophoresis on a 20% denaturing polyacrylamide gel followed by autoradiography. The bracket on the left of the autoradiogram denotes products considered to be elongated (E) RNA. The position of the molecular mass markers (NTs) are indicated by the numbers 2, 3, and 5 (representing GpG, GpGpU and GpGpUpCpU, respectively) on the left of the autoradiogram. The 7 and 9 indicate the positions of random oligodeoxyribonucleotide markers (Sigma Inc., St. Louis, MO). (B) Reaction products were quantified by scintillation counting of excised gel fragments. Solid circles indicate incorporation into E-RNAs, while open circles represent incorporation into the oligonucleotide of 8 bases. The amount of incorporation from the reaction at the last time point was arbitrarily taken as 100%. (C) The production of 8-mer is not due to limiting amounts of CTP. Products from a standard RdRp reaction (lane 1) and those to which unlabeled CTP was added to the final concentrations indicated on the top of the autoradiogram were analyzed as in (A). (D) Reaction products from the experiment shown in C were quantified by scintillation counting; incorporation from the standard reaction products (lane 1) were set at 100%.

amount of incorporation was observed with T7C and the 60S rRNA (Fig. 2 lanes 2 and 4), but only in the presence of BMV virion RNAs was the 8-mer generated (Fig. 2, lane 5). These results demonstrate that the BMV RdRp is responsible for synthesis of the 8-mer in a manner dependent on and specific to BMV virion RNA.

Effect of inhibitors on 8-mer and (−)-strand RNA synthesis

To further investigate whether the RdRp is responsible for the production of the 8-mer, we analyzed the effect of several inhibitors of DNA-dependent RNA polymerases on the reaction: actinomycin D, rifampicin, novobiocin, tagetitoxin, and heparin (Bautz, 1976; Johnson and McClure, 1976; Losick and Chamberlin, 1976; Mathews and Durbin, 1994; Webb and Jacob, 1988). Actinomycin D and rifampicin did not affect RdRp activity (Hardy *et al.,* 1979, and Fig. 3C), while the effects of novobiocin, tagetitoxin, and heparin were previously undescribed. We found that the latter three inhibitors decreased the ability of the BMV RdRp to synthesize the full-length (−)-strand RNA (Fig. 3 and data not shown). Tagetitoxin,

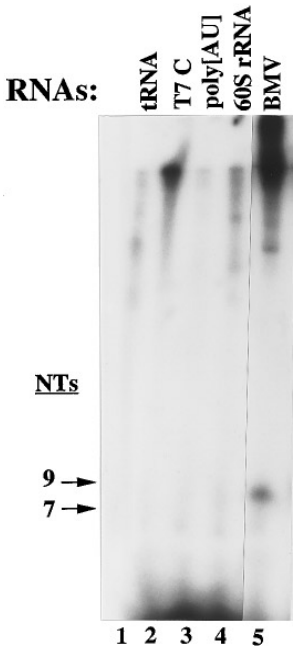

FIG. 2. BMV RNA-dependent and specific 8-mer generation. Autoradiogram of 20% denaturing polyacrylamide gel of products from RdRp reactions performed using the RNA templates (1 μg) indicated above the autoradiogram. BMV denotes RNA purified from BMV virions. The positions of 7- and 9-nucleotide (NTs) markers are indicated on the left of the autoradiogram.

known to be a potent inhibitor of bacterial RNA polymerases (Mathews and Durbin, 1994), reduced the activity of the *E. coli* RNA polymerase to 20% when present at 10 units per reaction (data not shown; units are defined by the manufacturer, Epicentre Technology Inc.). However, significant effects of tagetitoxin were only observed when used at 40 units (30% of the RdRp activity remained, data not shown). The need for such high concentrations of tagetitoxin precluded its use in further experiments. Novobiocin and heparin both inhibited the activity of the BMV RdRp in a dose-dependent manner (Figs. 3A and 3B). At 50 μM, novobiocin reduced activity to about 30% of control reactions. Heparin at 100 ng per reaction of 40 μl reduced RdRp activity to 10% of the control (Fig. 3B). These results identified three additional inhibitors of the BMV RdRp.

With regard to the synthesis of the 8-mer, actinomycin D and rifampicin had no significant effects (Fig. 3C, lanes 2–7). Novobiocin and heparin, however, inhibited the production the 8-mer (Fig. 3C, lanes 8–10, and Fig. 4A). Therefore, there is a consistent correlation between RdRp activity and the generation of the 8-mer.

Heparin is believed to interfere with polymerase recognition of template and hence blocks the initiation of transcription (Carpousis and Gralla, 1985; Pfeffer *et al.,* 1977). Inclusion of heparin in RdRp reactions resulted in decreases of both E-RNAs and the 8-mer (Fig. 4A). Quantification showed that the 8-mer was more sensitive to heparin than E-RNAs (Fig. 4B). Consequently, the molar ratio of the 8-mer to E-RNAs decreased along with the increase in heparin concentration (Fig. 4C). This results suggests that heparin inhibits (−)-strand RNA synthesis at the initiation stage, as represented by the 8-mer. However, polymerase complexes that somehow passed the initiation stage are less sensitive to the effects of heparin.

Characterization of nucleotide content of the 8-mer

Correct initiation of (−)-strand RNA synthesis has been shown to start at the penultimate C on the BMV virion RNA which ends with the sequence: 5′ . . . AAGAGA-C$\underline{C}$A$_{OH}$-3′ (the penultimate C is underlined (Kao and Sun, 1996; Miller *et al.,* 1986)). An 8-mer resulting from the correct initiation of RNA synthesis from this template should have the sequence 5′-GGUCUCUU-3′. Since this sequence lacks an adenylate, we determined whether the 8-mer contains this sequence by performing RdRp reactions using different radionucleotides. As indicated in Fig. 5A, the use of [^{32}P]CTP and [^{32}P]UTP gave readily detectable amounts of the 8-mer (lanes 3 and 4). The predicted sequence of the 8-mer should have four uridylates and only two cytidylates. Consistent with this prediction, quantification of the 8-mer labeled with [^{32}P]UTP and with [^{32}P]CTP revealed a ratio of 2:1. Reactions performed with [^{32}P]ATP resulted in the appearance of abundant amounts of elongated products, but not the 8-mer (Fig. 5, lane 1). Also apparent in this reaction are faint bands corresponding to lengths of 11 and 13 nucleotides (indicated by arrows in Fig. 5A). At positions -12 and -14 of the template RNA are two uridylates which will allow the formation of oligonucleotides of 11 and 13 nucleotides in length. The faint products could be due to termination of RNA synthesis due to limiting amounts of ATP in the reaction.

RdRp reactions performed with [^{32}P]GTP resulted in very few radiolabeled products (Fig. 5A, lane 2). A likely explanation is that RdRp needs ca. 50 μM of GTP to half-saturate the initiating nucleotide binding site (Kao and Sun, 1996); initiation in the reaction containing [^{32}P]GTP was unlikely due to the less than 1 μM of GTP present. In order to confirm that the 8-mer contains a guanylate, we performed reactions excluding one or more nucleotides. These reactions were predicted to produce only oligonucleotides and not E-RNAs. Reactions lacking ATP generated the 8-mer and lesser amounts of the 9-mer due to the use of radiolabeled [^{32}P]UTP (Fig. 5B, lane 4), confirming the previous hypothesis that adenylate is absent in the 8-mer. However, reactions lacking both ATP and GTP (Fig. 5B, lane 3) or lacking only GTP (lane 2) precluded the generation of the 8-mer, indicating that GTP must be present within the 8-mer. In addition, reactions performed with the diribonucleotide primer 5′-GpG-3′ (which should substitute for the two guanylates in the 8-mer) resulted in production of the 8-mer in the absence

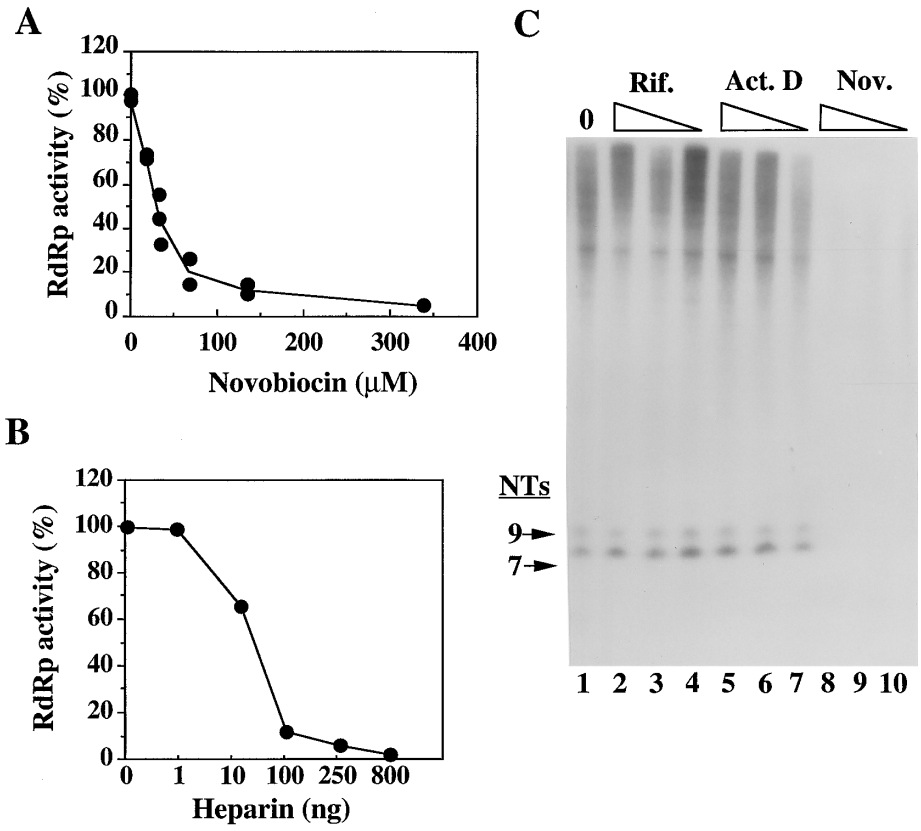

FIG. 3. Effects of RNA polymerase inhibitors on synthesis of full-length and 8-mer RNAs. Reactions were conducted by using the standard protocol described under Materials and Methods with the inclusion of inhibitors. Full-length RNA products were resolved by 1% agarose gel electrophoresis and were quantified by use of a Phosphorimager as described under Materials and Methods. (A) Effect of novobiocin (0, 18, 35, 68, 135, and 338 μM) on the synthesis of full-length (−)-strand RNA. (B) Effect of heparin (0, 1, 10, 100, 250, and 800 ng) on the synthesis of full-length (−)-strand RNAs. (C) Autoradiogram of results from a 20% denaturing polyacrylamide gel from RdRp reactions testing the effects of adding rifampicin, actinomycin, and novobiocin. Reactions were conducted by using the standard protocol described under Materials and Methods except that 0.30 μM [α-^{32}P]UTP was used, which resulted in the 8-mer and an additional oligonucleotide of 9 nucleotides. Results were quantified by liquid scintillation counting of razor blade-excised bands. Inhibitors were added at the following concentrations, rifampicin (Rif, 488, 244, 122 μM); actinomycin D (Act.D, 320, 160, and 80 μM); novobiocin (Nov., 200, 100, and 50 μM).

of GTP (data not shown). Altogether, these results show that production of the 8-mer requires UTP, CTP, and GTP, but not ATP.

An 8-mer initiated from the penultimate cytidylate at the 3′ end of BMV RNAs should contain two guanylates at its 5′ end. Digestion of the 8-mer with the guanosine-specific RNase T1 should then generate a 6-mer. To confirm this prediction, T7 RNA polymerase was used to synthesize an 8-mer (GGUCUCUU) as a positive control (see Materials and Methods). Lower-molecular-weight bands in the T7 transcription reaction represent the expected T7 polymerase abortive products (Fig. 5C, lane 1). The 8-mer produced by T7 RNA polymerase comigrated with the 8-mer generated by the RdRp (Fig. 5C, lanes 1 and 2), further confirming the size of the RdRp generated 8-mer. RNase T1 digestions of the 8-mer produced by T7 and by RdRp were performed using 2 units of enzyme for 20 min. The T7 RNA polymerase-synthesized 8-mers treated with RNase T1 generated a 6-mer with the expected sequence of 5′ UCUCUU 3′ (Fig. 5C, lane 4). Digestion of the 8-mer produced by the BMV RdRp also resulted in a 6-mer (Fig. 5C, lane 3). Taken together, the above three independent lines of evidence suggest that the 8-mer produced by the BMV RdRp contains the sequence, 5′-GGUCUCUU-3′. Therefore, we designated this 8-mer RNA an initiation product of the BMV RdRp.

Effects of Mn^{2+} on the formation of initiation product

We tested the effects of adding potassium chloride, ammonium sulfate, $MnCl_2$, or $MnSO_4$ to our standard RdRp reactions (containing 10 mM $MgCl_2$). Ammonium sulfate and potassium chloride both inhibited RdRp activity when present in the reaction at greater than 50 mM concentration. However, they did not change the molar ratio of the 8-mer to E-RNAs (data not shown). The presence of Mn^{2+} in the form of either $MnCl_2$ or $MnSO_4$ caused a reproducible increase in RNA synthesis. This increase was observed with up to 1 mM Mn^{2+}.

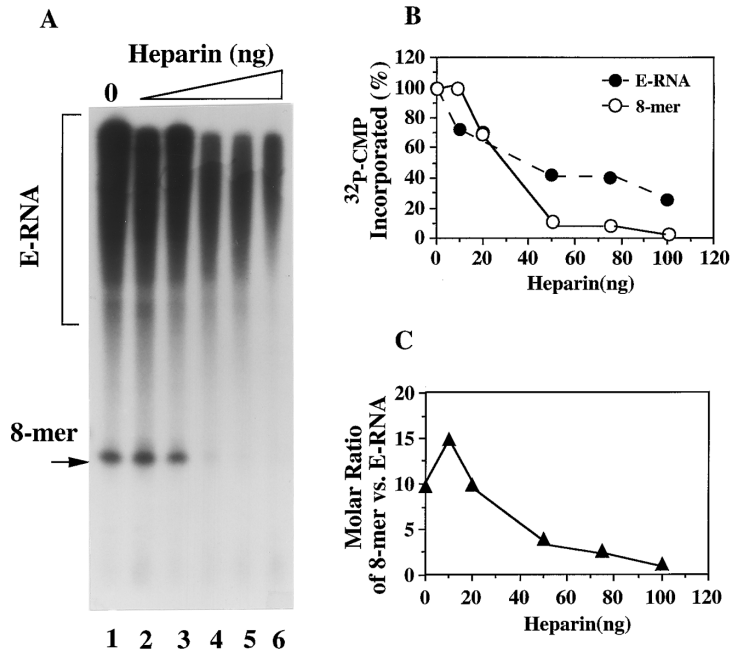

FIG. 4. Effect of heparin on the synthesis of 8-mer and E-RNAs. Reactions were conducted by using the standard protocol, analyzed by denaturing 20% polyacrylamide gels and quantified by liquid scintillation counting. (A) Autoradiogram demonstrating the effect of heparin added to the reactions at 0, 10, 20, 30, 50, and 100 ng (lanes 1 to 6, respectively). The bracket on the left of the autoradiogram denotes the region of the gel excised and counted as E-RNA. (B) Quantified results of the autoradiogram from A. Solid circles represent the amount of E-RNAs; open circles represent amounts of the 8-mer. (C) The calculated ratio of the 8-mer to E-RNA from the results in A.

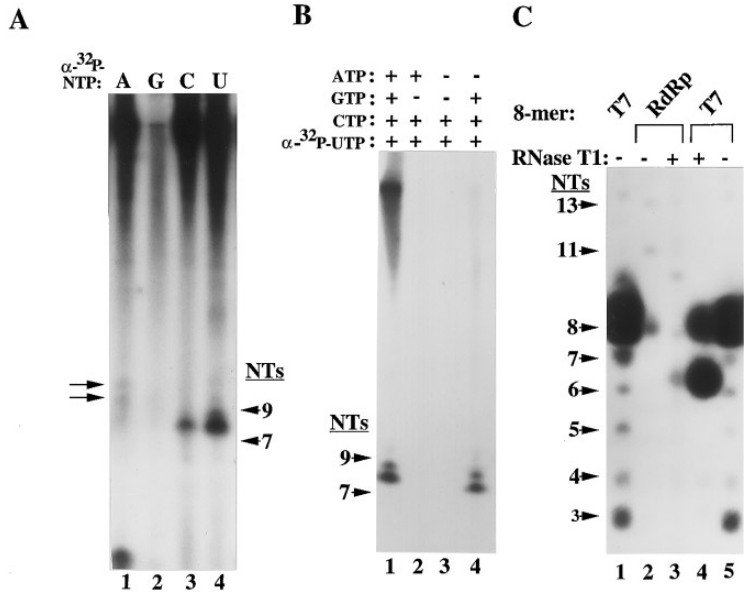

FIG. 5. Characterization of the sequence of the 8-mer. (A) Radiolabeling of the 8-mer. Reactions were conducted by using the standard protocol described under Materials and Methods except that NTP concentrations were adjusted to 15 μM ATP, 50 μM GTP, 3 μM CTP, and 3 μM UTP. The radiolabel used in each reaction is indicated on the top of the autoradiogram. For reactions with a particular radiolabeled nucleotide, the corresponding unlabeled nucleotide was omitted. (B) Guanosine is present within the sequence of the 8-mer. Combinations of NTPs were omitted as indicated above the autoradiogram and 0.3 μM [α ^{32}P]UTP was used as the radiolabel. (C) RNase T1 can digest the 8-mer produced both by RdRp and by T7. The T7 8-mer (5′-GGUCUCUU-3′) was generated by T7 polymerase as described under Materials and Methods. The RNAs were either untreated or treated with 2 units of RNase T1 for 20 min at 30° in 5-μl reactions and then separated on 20% denaturing polyacrylamide (15 $\times$ 42 cm; 800 V for 16 hr). Under these conditions, abortive products from the T7 polymerase reaction were separated into a ladder which included oligonucleotides of 4, 5, 6, 7, and 8 nucleotides in length. 11- and 13-mer positions were assigned based on this size ladder. Abortive products of T7 polymerase less than 3 nucleotides long will not be radiolabeled by the [α-^{32}P]UTP used in this reaction.

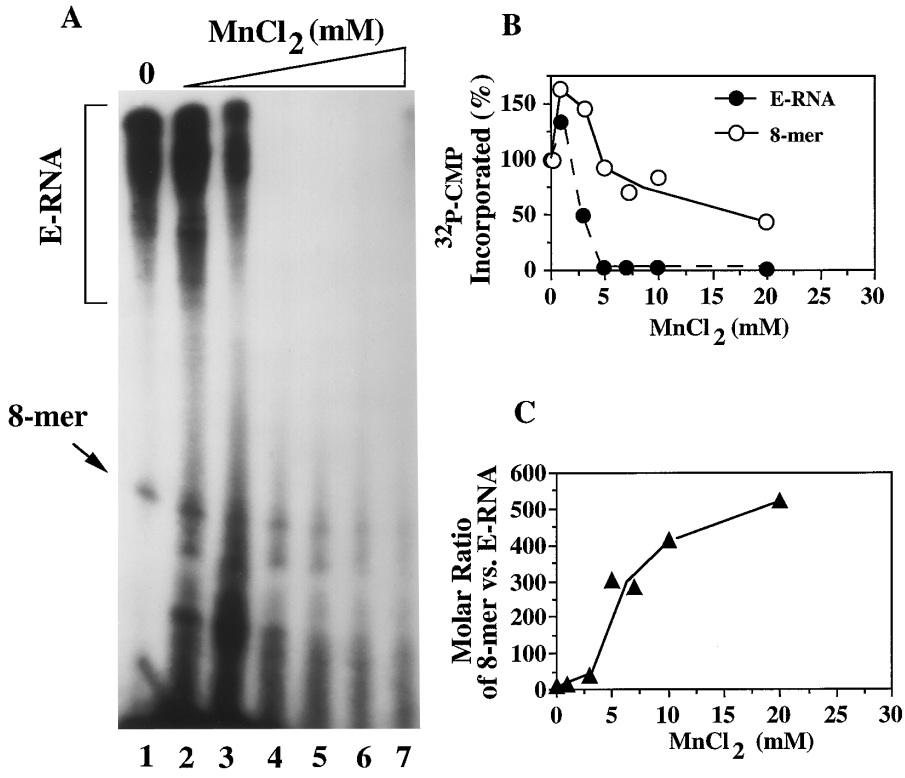

FIG. 6. Effects of Mn^{2+} on the accumulation of 8-mer and E-RNA. Reactions were conducted by using the standard protocol, analyzed by denaturing 20% polyacrylamide gels, and quantified by liquid scintillation counting. (A) The effects of including in RdRp reactions $MnCl_2$ at the final concentrations of 0, 1, 3, 5, 7, 10, and 20 m*M* (lanes 1 to 7, respectively). The 8-mer is indicated by an arrow and the region of the gel excised and quantified as E-RNA is denoted on the left by a bracket. (B) Quantified results from A. Solid circles represent the amount of E-RNAs; open circles represent amounts of the 8-mer. (C) Ratio of the amounts of the 8-mer and E-RNA from the experiment in A.

However, further increases of Mn^{2+} resulted in a marked inhibition of (−)-strand RNA synthesis (Figs. 6A and 6B). Quite interestingly, the inhibition of the production of E-RNAs was more pronounced than that of the production of the 8-mer and other shorter oligoribonucleotides. Starting at 3 m*M* Mn^{2+}, the E-RNAs were reduced in comparison to reactions lacking Mn^{2+}. However, severalfold higher than normal amounts of the 8-mer were generated along with additional oligoribonucleotides of smaller length (Fig. 6A, lane 3). Using 10 m*M* $MnCl_2$, the elongated product was completely abolished while significant amounts of the oligoribonucleotides remained (Fig. 6A, lane 6). Quantitation of results is presented in Fig. 6B, and the molar ratios of the 8-mer versus E-RNAs are presented in Fig. 6C. These results indicate that the presence of Mn^{2+} prevented the elongation of nascent RNA.

In order to examine the effects of Mn^{2+} on the production of the 8-mer in reactions lacking Mg^{2+}, RdRp preparations were passed through a gel-filtration column in buffer lacking Mg^{2+} and the effect of the addition of Mg^{2+} and Mn^{2+} was examined. Reactions without additional divalent metal retained less than 10% of the initial activity (Fig. 7A, lanes 1 and 9), indicating that Mg^{2+} is indeed limiting. Addition of $MgCl_2$ to this reaction system restored RNA synthesis of both the E- and 8-mer RNAs (Fig. 7A, lanes 2–8; Fig. 7B). When 1 to 5 m*M* Mn^{2+} was added to the same enzyme preparation, synthesis of E-RNAs was not observed, but the level of the 8-mer and other smaller oligoribonucleotides increased dramatically (Fig. 7A, lanes 10–13; Fig. 7C). Higher concentration of Mn^{2+} had an inhibitory effect on synthesis of oligonucleotides (Fig. 7A, lanes 14 and 15). These results indicate that 1 to 5 m*M* Mn^{2+} was able to replace Mg^{2+} and allow initiation of (−)-strand RNA synthesis. However, Mn^{2+} cannot replace Mg^{2+} for subsequent steps.

Effects of sequences at 3′ end of template on initiation and elongation

To determine whether the sequence context near the 3′ end of BMV RNA affects initiation of (−)-strand RNA synthesis, we examined the effects of mutations and extensions at the 3′ end on synthesis of the 8-mer and elongated products (Fig. 8A). Mutations included an RNA with deletion of the 3′-terminal A (Δ1), RNAs with extensions at their 3′ ends (+1G, +GAU, +13, and +17), and RNAs with single nucleotide changes (mu-1, -3, -4). The substitutions in the mutant templates mu-3 and mu-4 contain transitions and do not alter the amounts of pyrimi-

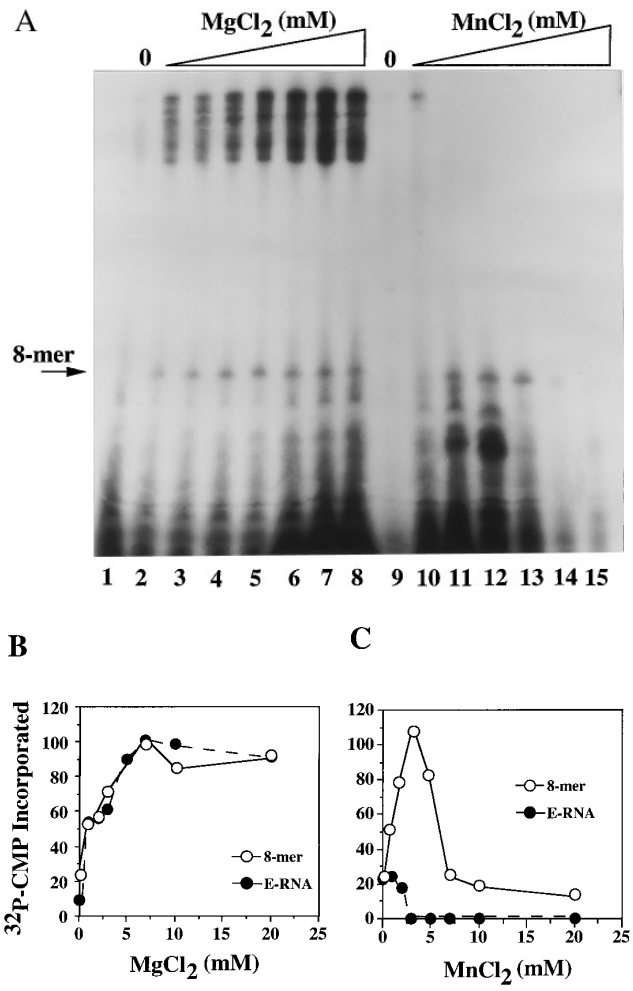

FIG. 7. Effects of Mn^{2+} and Mg^{2+} on the production of the 8-mer and E-RNA. RdRp (8.6 μg protein) lacking Mg^{2+} was prepared by gel-filtration chromatography. The RdRp was loaded onto a PD-10 column (Pharmacia Biotech) equilibrated with buffer (pH 8.2) containing 50 m*M* Tris–HCl, 50 m*M* KCl, 2 m*M* DTT, 25% glycerol, and 0.75% Triton X-100 and eluted with the same buffer. The reactions were amended with either Mg^{2+} (lanes 2–8) or Mn^{2+} (lanes 10–15) as indicated above the autoradiogram. The final concentration of divalent metal are 0, 1, 2, 3, 5, 7, 10, and 20 m*M* (lanes 1–8 and 9–15, respectively). Reaction products were resolved on the 20% denaturing PAGE (A). The 8-mer and E-RNA were quantified by liquid scintillation counting and the results for Mg^{2+} and Mn^{2+} are presented in B and C, respectively. Solid circles represent amount of E-RNAs. Open circles represent counts of 8-mer.

dines and purines. Mutant mu-1, in which the 3′ terminal A is changed to a C, was examined previously by Dreher *et al.* (1984).

In vitro transcribed BMV RNA3 served as template for the synthesis of both E-RNAs and the 8-mer (Fig. 8B, lanes 5, 16, and 17). Consistent with earlier results, wild-type RNA3 produced approximately 10-fold higher molar amounts of the 8-mer than elongated (−)-strand RNA (Table 1). The transcripts are free of contaminating NTPs or other inhibitors as assayed by incorporation of radiolabeled CMP into transcripts produced by T7 RNA polymerase (data not shown).

While all the mutant templates decreased the amount of 8-mer synthesis, they had different effects on the synthesis of E-RNAs (Fig. 8C) and on the ratio of E-RNAs and the 8-mer (Table 1 and Fig. 8B, lanes 3, 4, 8–15). Extensions at the end of the template RNA had several interesting effects. Addition of a guanylate to the 3′ end and deletion of the terminal adenylate decreased the amount of both the 8-mer and E-RNAs, but did not significantly alter the ratio of the products. In contrast, additions of 3, 13, or 17 nucleotides decreased the amount of 8-mer produced while increasing the production of elongated products (Table 1). Coupled with the reduced amounts of 8-mer produced from these templates, the ratio of the 8-mer to elongated products for all three templates was severalfold lower than that for the wildtype template.

Substitution of the terminal A with C decreased not only the 8-mer and E-RNAs synthesized, but also the molar ratio from 10- to 4-fold (Table 1), indicating a more severe effect on the synthesis of the 8-mer than on E-RNAs. Substitution of C at the -3 position with U or substi-

tution of the A at the -4 position with G decreased both the 8-mer and E-RNA synthesis. However, E-RNA synthesis was more severely decreased, resulting in a slight

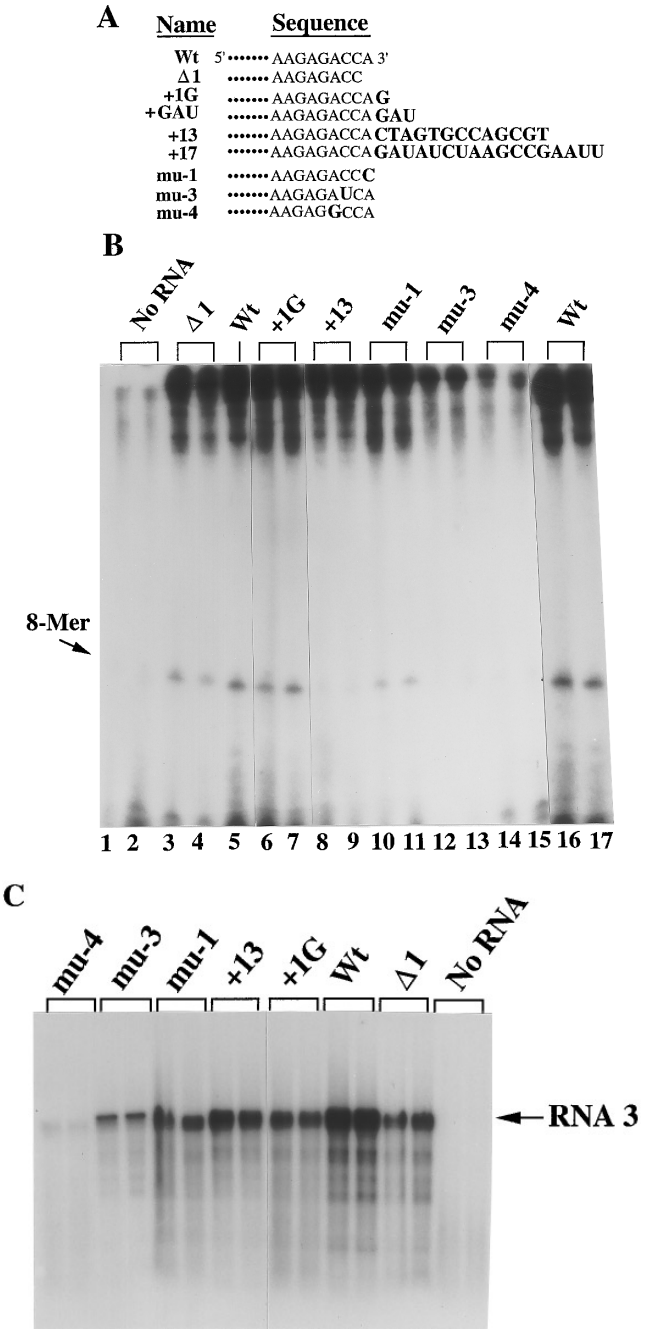

FIG. 8. Transcription activity of the BMV RdRp on the (+)-strand RNA3 template with altered 3′ ends. (A) Schematic of the 3′ end of RNA templates used in these experiments. (B) Autoradiogram of the products synthesized by the BMV RdRp using the indicated templates resolved on a 20% denaturing polyacrylamide gel. For most of the reactions, two independent samples for each transcript were performed in this experiment and both reactions are shown in the autoradiogram. The position of the 8-mer is indicated on the left. Products for transcripts +GAU and +17 nucleotides were tested in a separate experiment (data not shown). (C) Aliquots of the products of the reactions shown in B were also resolved on a 1% agarose gel.

TABLE 1

Effects of Different Template RNA 3′ Ends on Initiation and Elongation

RNA tested[a]	8-mer (%)[b]	E-RNA (%)[b]	Molar ratio of 8-mer/E-RNA[b]
Wt	100 ± 21 (n = 12)[c]	100 ± 12	10.0 ± 4.1
Δ1	29 ± 7 (n = 4)	49 ± 8	8.6 ± 2.0
+1G	52 ± 8 (n = 4)	57 ± 13	11.6 ± 2.5
+GAU	21 ± 15 (n = 4)	101 ± 29	2.7 ± 1.7
+13	20 ± 17 (n = 12)	139 ± 27	1.8 ± 1.6
+17	53 ± 13 (n = 4)	176 ± 18	3.3 ± 0.7
mu-1	14 ± 7 (n = 4)	46 ± 8	3.9 ± 2.1
mu-3	35 ± 8 (n = 4)	31 ± 9	12.3 ± 6.0
mu-4	40 ± 13 (n = 4)	21 ± 5	14.7 ± 5.5

[a] The sequences for RNA tested were presented in Fig. 8A.

[b] Quantification of the 8-mer, the E-RNA, and the molar ratio were described under Materials and Methods. The numbers were converted to percentage of Wt.

[c] n, number of independent trials used to determine the amount of products produced by RdRp for each template. Values after the ± denote the standard deviation.

increase in the molar ratio of the two products. These results indicate that the sequence context near the RNA initiation site can affect the ability of RdRp to initiate RNA synthesis and elongation.

DISCUSSION

RNA synthesis by the BMV RdRp is highly template specific (Hardy *et al.,* 1979). Until recently, characterization of the BMV RdRp has relied primarily on the detection of (−)-strand RNA corresponding to the size of template RNA. We have recently examined the initiation of (−)-strand RNA synthesis using oligonucleotides as primers (Kao and Sun, 1996). We now extend these studies and demonstrate the existence of a predominant initiation product of 8 nucleotides synthesized from (−)-strand RNA. The 8-mer is produced in a template-dependent manner and is correctly initiated from (−)-strand RNA templates, as evidenced by its nucleotide content and its dependency on the high concentration of GTP or GpG. Furthermore, changes in the sequence near the initiation site on the template had an effect on the molar ratio of the 8-mer to E-RNA (Table 1). Finally, the relative stoichiometry of the 8-mer is about 10-fold higher than that of the E-RNA. Altogether, these results suggest that the BMV RdRp undergoes abortive initiation during the synthesis of (−)-strand RNA.

Using previous studies of DNA-dependent RNA polymerases as models, it is possible to divide the (−)-strand RNA synthesis from BMV virion RNA into multiple steps. The efficiency of RNA synthesis could be affected by two key regulatory processes: (1) the interaction of the polymerase with the template and (2) a competition between productive elongation and dissociation of the initi-

ation complexes. The characterization of the initiation products produced by RdRp will allow analysis of RNA synthesis by RdRp as a multistep process.

Since the 8-mer is present in 10-fold molar excess in comparison to E-RNA, dissociation of the initiation complex is a more common phenomenon than progression into the stable elongation complex. Therefore, an additional event after the polymerization of 8 nucleotides must lead to the formation of a more stable ternary complex. As indicated in Fig. 8B and by studies using T7 RNA polymerase (Martin *et al.,* 1988), the extent of this dissociation depends on the sequence context near the initiation region. Deletion of the terminal A resulted in reduction of both initiation and elongation products without affecting their molar ratio (Fig. 8B and Table 1). This result suggests that the terminal adenylate can affect the interaction between RdRp and RNA and its removal can hamper the entire process of RNA synthesis. Substitution of the terminal adenylate with a cytidylate had a more profound effect on the generation of the initiation products and a lesser effect on elongation as evidenced by a decrease in the molar ratio of initiation products to elongation products (Fig. 8B and Table 1). The terminal adenylate appears to influence the initiation process even though it is not used to direct the incorporation of the initiating nucleotide. Deletion of the 3′-most CA or its substitution by CC, CU, AC, or UU resulted in a marked decrease in (−)-strand RNA synthesis (Dreher *et al.,* 1984; Miller *et al.,* 1986). Substitutions at -3 and -4 had more significant effects on the E-RNA synthesis than initiation (Table 1). These results better define the interaction between RdRp and the sequence required for initiation and elongation of RNA synthesis.

An unexpected result of this work is that extensions of more than 3 nucleotides at the 3′ end of the template RNA favored the synthesis of E-RNAs at the expense of 8-mer synthesis. For a wildtype RNA, initiation of RNA synthesis by RdRp could be a physically difficult task for RdRp because of the proximity to the free 3′ end of the template. The extensions could provide a scaffold for more stable RdRp binding, hence promote progression into the elongation mode. It should be noted that the extensions of 13 and 17 bases were of different sequences. However, the sequence of the extension can affect initiation, since we have observed that an extension of only three guanylates will decrease the utilization of the template by 90% (Sun, Faurote, and Kao, unpublished results). This observation likely explains the difference between our result and the previous report of Miller *et al.* (1986), who observed that a 15-nucleotide extension severely decreased the synthesis of (−)-strand RNA.

The identity of divalent metal has a striking effect on the ratio of initiation versus elongation products. Several previous studies, including those using chloroplast RNA polymerase, report that the abortive initiation products are Mn^{2+}-dependent (Job *et al.,* 1987; Mercoyrol *et al.,* 1990; Mosig *et al.,* 1985). Job *et al.* (1987) also observed a Mn^{2+}-induced increase in generation of abortive products by the wheat germ RNA polymerase II and *E. coli* RNA polymerase. Mosig *et al.,* (1985) found that primer-independent abortive initiation catalyzed by wheat germ RNA polymerase II had a highly increased rate in the presence of Mn^{2+}, rather than Mg^{2+}. Using the BMV RdRp, the 8-mer is produced in the presence of Mg^{2+}, but its presence is enhanced by Mn^{2+}. Quite strikingly, the 8-mer can be produced in a Mn^{2+}-dependent manner, but elongation requires the presence of Mg^{2+} in the reaction (Fig. 6A). Mg^{2+} may therefore play some role in stabilizing the elongation complex.

The chloroplast RNA polymerase (Job *et al.,* 1987) and wheat germ RNA polymerase II (Mercoyrol *et al.,* 1990) are also not active for the elongation process in the presence of Mn^{2+}. In addition, Mg^{2+} strongly alters the catalytic properties of the elongation complex and may stabilize the ternary complex (a complex of polymerase, template, and newly initiated RNA). Therefore, inhibition of E-RNA synthesis resulting from the addition of Mn^{2+} to the RdRp activity assay might be due to the competition of Mn^{2+} with Mg^{2+} for access to the catalytic active site, resulting in a labile ternary polymerase complex. In agreement with this interpretation, we observed an increase in the amount of shorter abortive products in reactions containing Mn^{2+} (Figs. 6C and 7A). Woody *et al.* (1996) have modeled the geometric conformation of Mn^{2+} and Mg^{2+} in complex with the catalytic site of T7 RNA polymerase and have evidence that this geometry plays a role in catalytic activity.

Abortive cycling is a normal part of transcription from a DNA template *in vitro* (Krummel and Chamberlin, 1992; Martin *et al.,* 1988; Matsumoto, 1994). Little is known about the RNA synthesis from an RNA template occurring in an RNA virus. Our work is the first to demonstrate that abortive cycling occurs during RNA synthesis by an RdRp encoded by a (+)-strand virus. Further efforts to study the abortive initiation products are required to provide more insights into the mechanism of RNA replication and the interaction between RdRp and the template RNA. Characterization of the putative abortive initiation products and the observation that heparin and Mn^{2+} have different effects on the formation of the initiation and elongation products will aid in the development of assays to further study the transition from initiation to the elongation that occurs during (−)-strand RNA synthesis from viral RNA. Finally, the mechanism of initiation (Kao and Sun, 1996) and the synthesis of abortive initiation products reported here strongly suggest that the BMV RdRp undergoes a repertoire of steps very similar to that of the DNA-dependent RNA polymerases.

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